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Liposomes are effective carriers for the ocular delivery of prophylactics

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Liposomes containing acetylcholinesterase were prepared by the freeze-drying method. The multilamellar morphology of the vesicles was revealed by freeze-fracture electron microscopy and their size distribution was determined by quasi-elastic light scattering. The vesicle diameters were in the range of about 0.2–4.0 μm. The liposome preparations were tested for their ocular delivery of an entrapped cholinesterase enzyme in counteracting the miotic effect of diisopropylfluorophosphate (DFP), a prototype of a family of organophosphate poisons. The topical application of the enzyme-containing liposomes to the rabbit eye was found to confer a significant level of protection against DFP-induced miosis. In comparing the prophylactic effectiveness of different enzyme-bearing liposomes, positively charged vesicles were found to be more effective than either neutral or negatively charged vesicles. Although the precise protective mechanism is not clear, our in vitro studies indicate that DFP molecules freely associate with liposomes and tear fluid promotes the release of liposome-entrapped enzymes. Thus, it is conceivable that the enzyme-liposome complex may act somewhat like a sponge by sequestering DFP molecules which diffuse into the vesicle, and also by releasing the entrapped enzyme to combine with DFP, thereby neutralizing its in vivo toxic effect.

Introduction

Liposomes, first described by Bangham [1], are biocompatible and biodegradable phospholipid microcapsules which have been considered excellent candidates for the encapsulation and delivery of biological and pharmacological agents [2]. Whereas the delivery of entrapped agents to target cells in vitro has been achieved [3–5], the intravenous targeting of liposomes to extravascular and nonreticuloendothelial sites has not met with particular success [6]. The topical application of liposomes for the delivery of entrapped agents to selected anatomical locations, however, appears promising because the problem of active targeting is bypassed. By applying the liposome formulation directly to or in the vicinity of the target, the probability of a premature release of the entrapped agent is minimized [7].

The incorporation of drugs into liposomes has been reported to alter drug bioavailability in ocular tissues [8]. Liposomes can enhance or reduce the ocular absorption of encapsulated agents applied to the eye. The nature and extent of altered ocular uptake of liposome-associated agents appear to depend on a number of factors, e.g., physicochemical properties of the entrapped agent, chemical composition and physical characteristics of the liposomes used, and the method of ocular administration of the liposomal formulation [9].

Abbreviations: DFP, diisopropylfluorophosphate; PC, phosphatidylcholine; HDL, high-density lipoprotein.

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In this study, we examined the possibility of using liposomes for the ocular delivery of a prophylactic enzyme in counteracting the miotic effect of diisopropylfluorophosphate (DFP), a prototype of a family of organophosphate poisons found in insecticides and potential chemical warfare agents [10]. The potential exposure of these and other toxic agents to the eye constitutes serious hazards for some professional groups in the agricultural industry and the military. Liposomes containing acetylcholinesterase were prepared by the freeze-drying method and their morphological characteristics and size distribution determined. The liposomal preparations were tested for their prophylactic efficacy in counteracting DFP-induced miosis. The rationale for the in vivo testing is based on the neutralization of the toxic effect of DFP after its irreversible binding to acetylcholinesterase. Our data indicate that the instillation of liposomes containing the cholinesterase enzyme is effective in providing significant protection against DFP-induced miotic insults. A 'sponge hypothesis' was proposed to account for the prophylactic action of the enzyme-liposome complex.

Materials and Methods

Animals and tear collection. Normal female New Zealand white rabbits, 12–14 weeks old and 2.0–2.5 kg in weight, were used for ocular bioassays and for tear collections. Tear fluids were collected from the lower eyelid margins of rabbits with 10-μl micropipettes. Extreme care was taken to avoid irritating the eye so as not to stimulate tearing.

Chemicals. Egg phosphatidylcholine (PC), cholesterol, stearylamine, phosphatidic acid, acetylcholinesterase and DFP were obtained from Sigma Chemical Co. (St. Louis, MO). (1,3-^{3}H)-labeled DFP was purchased from New England Nuclear, Boston, MA.

Preparation of liposomes. Liposomes were prepared using the freeze-drying method described by Kirby and Gregoriadis [11]. A total of 66 μmol of lipids dissolved in chloroform/methanol (2:1, v/v) were dried in a thin film on the bottom of a screw-capped culture tube with a stream of nitrogen. Egg PC, cholesterol and stearylamine or phosphatidic acid were used in various combinations. The lipids were further dried in vacuo for 30 min, after which they were hydrated with 1 ml of 4 mM $NaHCO_3$ (pH 7.4). The samples were then vigorously vortexed at room temperature (20°C) and sonicated at 100 W for 3 min under N_2. Following this step, 1 ml of acetylcholinesterase (type V-S, Sigma) dissolved in distilled water at 2 mg/ml was added. After mixing, the samples were shell frozen and then freeze-dried overnight. On the day before use, the samples were rehydrated with 200 μl of distilled water at 5°C overnight. The reconstituted liposomes were then washed twice by pelleting in 38 ml of 4 mM $NaHCO_3$ at $10000 \times g$ for 20 min. The final pellet was taken up to 1 ml in 4 mM $NaHCO_3$ (pH 7.4) and enzyme activity was determined colorimetrically using a cholinesterase diagnostic kit (Sigma kit no. 420).

Electron microscopy. Freeze-fracture electron microscopy was used to examine the morphology of the liposome preparations. Liposome samples were quenched from room temperature by plunging into partially solidified Freon 22. The samples were freeze-fractured using a Balzers freeze-fracture unit at −100°C. Fractured samples were platinum shadowed at an angle of 45°. The carbon replicas were cleaned in concentrated bleach for about 30 min and then in chloroform/methanol (2:1, v/v) for a further 10–15 min. The replicas were examined using a Philips Model-300 transmission electron microscope.

Vesicle sizing. Quasi-elastic light scattering was used to determine the size distribution of the liposome preparation. A small aliquot of sample was diluted to 2 ml with distilled water and light scattering was recorded at an angle of 90° in a thermally jacketed sample-chamber maintained at 21.3°C. Measurements were made at three sample times using a helium-neon laser (wavelength 632.8 nm), a quantum photometer and a 64-channel autocorrelator (Langley-Ford model 1096). Analysis of the resulting autocorrelation functions was carried out using the method of cumulants.

Measurement of release of liposome-associated enzymes. The active site of acetylcholinesterase was radiolabelled by incubation with ^{3}H-labeled DFP. 1 mg of the enzyme was mixed with 5.74 nmol of ^{3}H-labeled DFP (specific activity, 3.0

Ci/mmol) in 2 ml of phosphate-buffered saline (20 mM phosphate buffer containing 1.0 M NaCl) (pH 7.0) and incubated at 20 °C for 90 min followed by dialysis against the phosphate-buffered saline for 48 h at 5 °C. Freeze-dried vesicles (PC/cholesterol/stearylamine, 7 : 2 : 1), consisting of 66 μmol of total lipid, were prepared as described earlier. After drying in vacuo, the sample was hydrated with a small volume of distilled water, vigorously votexed, and sonicated at 100 W for 3 min under N_2. To this, 1 mg of labelled enzyme ($1.6 \cdot 10^6$ cpm) and 1 mg of unlabelled enzyme, both in phosphate-buffered saline, were added and the final volume adjusted to 2.4 ml. After mixing, the sample was divided into three equal aliquots and freeze-dried overnight. Samples were stored under an atmosphere of N_2 at −20 °C until required.

For each release study, one aliquot was rehydrated overnight with 33 μl of distilled water at 5 °C under N_2. The preparations were washed three times in 8 ml of 10 mM phosphate/100 mM NaCl (pH 7.0) by ultracentrifugation at 110000 × *g* for 15 min at 5 °C. The final pellet was gently resuspended with 550 μl of buffer; 150-μl aliquots were immediately mixed with equal volumes of either rabbit tears or buffer. The mixtures were incubated at 37 °C for 15 min or 3 h and the released enzyme was separated from liposome-associated enzyme by flotation on a discontinuous density gradient. The gradient consisted of four phases (from bottom to top): 0.4 ml of 20% CsCl, 0.3 ml of sample made 20% in sucrose, 4 ml of 10% sucrose in buffer (10 mM phosphate/100 mM NaCl, pH 7.0), and 1 ml of the same buffer on top. The tubes were then centrifuged at 49000 rev./min in a Beckman SW-50.1 rotor for 2.5 h. The tube contents were fractionated and the activity of each recovered fraction was determined by liquid scintillation counting. The amount of released enzyme, recovered in the CsCl phase of the gradient, was expressed as a percentage of the initial total entrapment.

Measurement of changes in pupil size in rabbits. Each animal to be examined was gently hand-restrained on a table and the eye was illuminated at 2.5 mW/cm² by a rheostat-controlled incandescent light placed at a distance of 0.5 m away. Measurements of the pupil size were made as follows: A mm scale was positioned below the

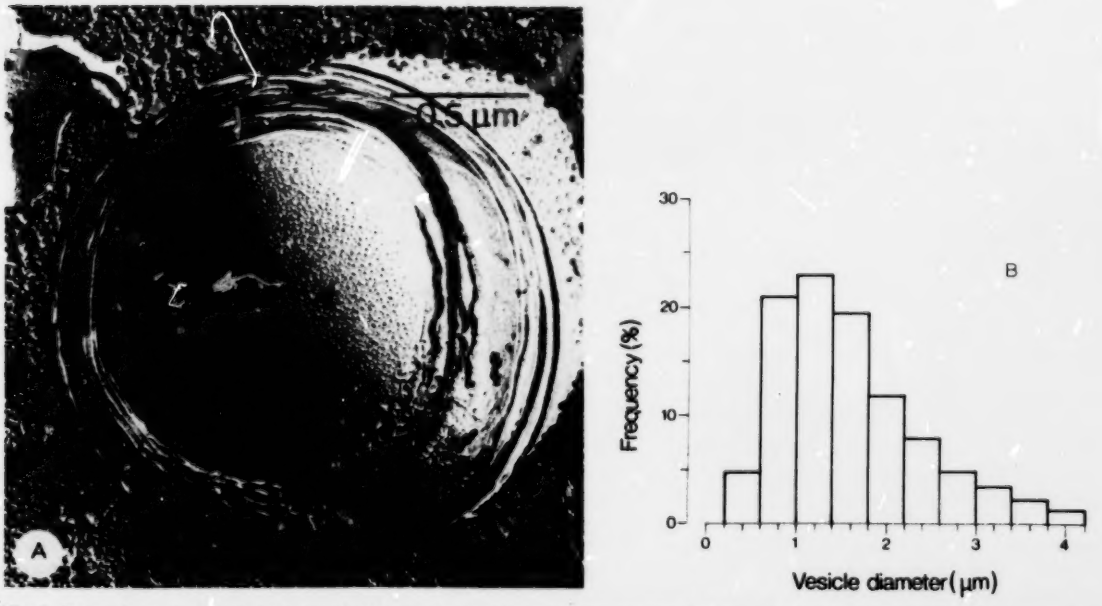

Fig. 1. Freeze-fracture electron micrograph (A) and size distribution histogram (B) of freeze-dried vesicles prepared in the presence of acetylcholinesterase. The hydrodynamic diameters of the vesicles were computed assuming they were spherical.

lower eye lid and photographs were taken with a Nikkormat single-lens-reflex camera fitted with a 135-mm lens and loaded with Kodak Ektachrome film (ASA 200). The aperture setting was $F/5.6$ at a shutter speed of 1 s. Data on the pupil size were determined by projection of the slides on a screen and measurements were made using the projected mm scale accompanying the picture of each eye.

Results

Electron microscopy and size distribution

Fig. 1 shows a freeze-fracture electron micrograph of a typical freeze-dried vesicle and a size distribution histogram. The multilamellar nature of the freeze-dried vesicle is deemed desirable for a more gradual and sustained release of the

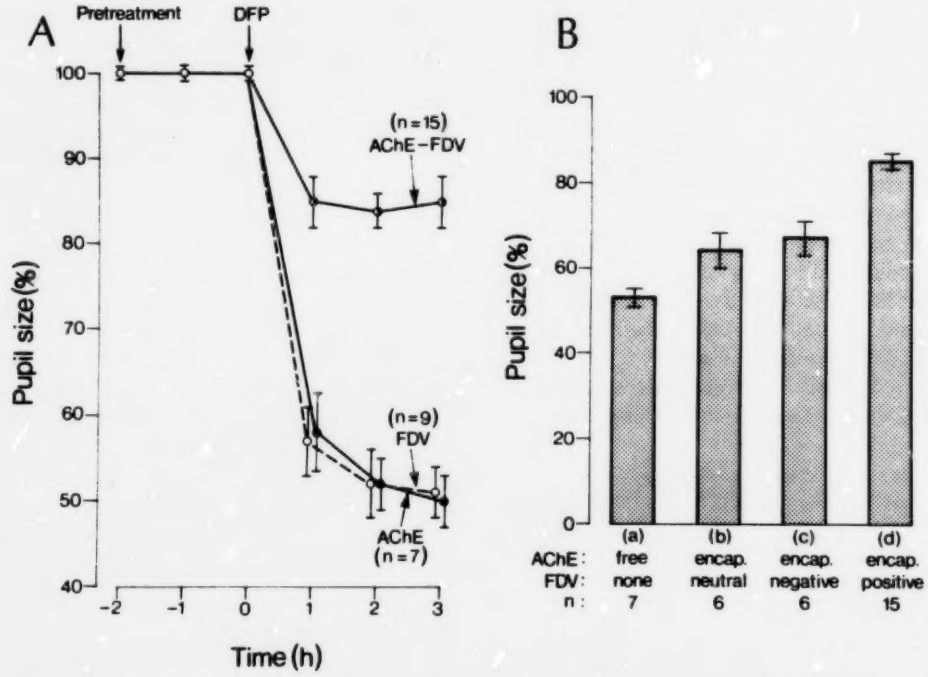

Fig. 2. Efficacy of freeze-dried vesicle-encapsulated acetylcholinesterase (AChE) in counteracting DFP-induced miosis. The procedure for the measurement of pupil size is described in Materials and Methods and the baseline pupil size was first determined prior to any treatment applied to the eye. (A) Pretreatment consisted of administering to the eye either enzyme-containing liposomes (AChE-FDV), free enzyme (AChE) or plain liposomes (FDV). The dose of acetylcholinesterase applied in each case contained 140 RU of enzyme activity and the lipid content of the freeze-dried vesicles (PC/cholesterol/stearylamine, 7:2:1) applied was 2.6 μmol. The topical application was performed by dropping the appropriate preparation (40 μl) on the cornea with the excess retained by the conjunctival sac. 2 h later, each eye was subjected to a miotic challenge by the instillation of an effective dose (4 μg in 20 μl) of DFP previously determined to reduce the pupil size by 50% (ED_{50}). Subsequent changes in the pupil size were monitored at hourly intervals. Each point in the figure represents the mean percentage of original pupil size, ±S.E., of the number of determinations indicated. (B) The effectiveness of acetylcholinesterase-containing liposomes of different surface charge in preventing DFP-induced miosis was determined. The pretreatment of the eye consisted of the instillation of either (a) free acetylcholinesterase solution, (b) acetylcholinesterase encapsulated in neutral freeze-dried vesicles (PC/cholesterol, 8:2) (c) acetylcholinesterase in negative freeze-dried vesicles (PC/cholesterol/phosphatidic acid, 7:2:1), or (d) acetylcholinesterase in positive freeze-dried vesicles (PC/cholesterol/stearylamine, 7:2:1). The dose of acetylcholinesterase applied to each eye was between 140 and 144 RU of enzyme activity and the freeze-dried vesicle lipid content was 2.6 μmol. 2 h after pretreatment, each eye was challenged with an ED_{50} of DFP. Each vertical bar represents the mean percentage of original pupil size ± S.E. of the number of determinations indicated in the figure. Comparison of the statistical significance (Student's *t*-test) between the mean values among different groups: a and b, $P < 0.02$; a and c, $P < 0.05$; a and d, $P < 0.0001$; b and c, not significant; b and d, $P < 0.0001$; c and d, $P < 0.003$.

entrapped acetylcholinesterase for prophylactic purpose. The size-frequency distribution of the acetylcholinesterase-containing freeze-dried vesicles indicates the presence of vesicles ranging in diameter from about 0.2 to 4 μm. The predominant freeze-dried vesicle population, however, is between 0.6 and 1.8 μm and the average diameter of all the vesicles is 1.7 μm.

Efficacy of liposome-associated acetylcholinesterase in counteracting DFP-induced miosis

Liposome-entrapped acetylcholinesterase, possessing 140 Rappaport units, RU [12], of enzyme activity, was applied to one eye of the rabbit 2 h before the instillation of DFP. The contralateral eye of the same animal served as the control and received an identical dose of acetylcholinesterase, but in free form. The effective dose of DFP applied to induce a reduction of the pupil size by 50% was predetermined to be 4 μg (ED_{50}). It can be seen from Fig. 2A that pretreatment with liposome-entrapped acetylcholinesterase significantly reduced DFP-induced miosis. On the other hand, the application of free acetylcholinesterase solution or plain liposomes did not provide any protection against a similar DFP challenge. It can be seen from Fig. 2B that positively charged vesicles (PC/cholesterol/stearylamine, 7:2:1) provided the best prophylactic effect compared with either neutral (PC/cholesterol, 8:2) or negatively charged vesicles (PC/cholesterol/phosphatidic acid, 7:2:1).

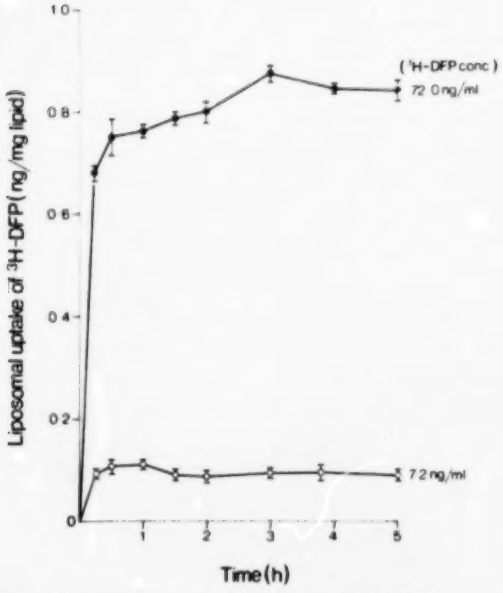

Fig. 3. Liposomal uptake of ^{3}H-labeled DFP. Multilamellar liposomes (PC/cholesterol/stearylamine, 7:2:1), consisting of 33 μmol of total lipid, were incubated with the indicated concentration of ^{3}H-labeled DFP (specific activity, 3.0 Ci/mmol) and 100-μl aliquots were taken at specified times to determine the extent of DFP-liposome association. Separation of liposome-bound from unbound ^{3}H-labeled DFP was accomplished by ultracentrifugation at 114000 × g for 15 min at 5°C. The distribution of labelled DFP in both the resulting pellet and the supernatant at the indicated time interval was determined by scintillation counting. Each point represents the mean liposomal uptake of ^{3}H-labeled DFP, ±S.E., of three separate experiments.

Liposomal uptake of DFP

To determine whether DFP can readily associate with the liposome, we allowed preformed vesicles to interact freely with tritiated DFP and followed the kinetics of its uptake (Fig. 3). Positively charged multilamellar liposomes were observed to rapidly take up labelled DFP at both concentrations used in this study, with equilibrium being established within 15 min at the lower concentration. While the data in Fig. 3 could indicate adsorption to or insertion into the outer vesicle bilayer without actual penetration into inner compartments, the reverse experiment in which we entrapped labelled DFP within freeze-dried vesicles of the same composition indicated rapid release of at least 50% of the label (data not shown).

Tear-induced release of liposome-entrapped enzyme

In the experiment shown in Fig. 4, we examined the effect of rabbit tear fluid on the fate of a liposome-entrapped enzyme. To follow the rate of possible enzyme release, tritiated DFP-labelled acetylcholinesterase was used for entrapment. This method of labelling acetylcholinesterase has been demonstrated to be useful in monitoring the extent of enzyme association with preformed lipid vesicles [13]. In the experiment described here, we followed the reverse situation, namely the release of labelled acetylcholinesterase after entrapment

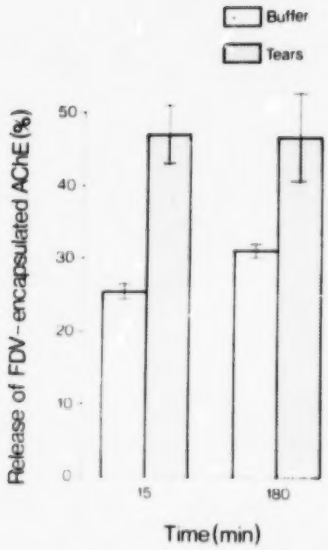

Fig. 4. Tear-induced release of freeze-dried vesicle-(FDV) associated acetylcholinesterase (AChE). The procedure for measuring the release of liposome-associated enzyme is described in Materials and Methods. Each vertical bar represents the mean release of freeze-dried vesicle-encapsulated acetylcholinesterase, ± S.E., of three separate experiments. The difference between buffer- and tear-induced enzyme release after 15 or 180 min of incubation is statistically significant (Student's *t*-test) in each case, $P < 0.005$ and $P < 0.05$, respectively.

in liposomes. It can be seen in Fig. 4 that there is a rapid and limited release of acetylcholinesterase in the presence of rabbit tears which is complete within 15 min of exposure. Subsequent incubation with tear fluid for a period of 3 h results in no further release.

Discussion

A number of methods are available for the preparation of different types of liposomes [14]. The preparative procedures can be adapted to suit specific needs and, in this study, freeze-dried vesicles were used for the entrapment of acetylcholinesterase. The freeze-drying method was chosen because of its simplicity and, more importantly, because during preparation the enzyme is at no time exposed to organic solvents or sonic energy, both of which were found to destroy the enzyme's activity completely. Thus, certain liposome-preparation procedures, e.g., reverse-phase evaporation [15] and solvent injection [16,17] methods, are inappropriate for the entrapment of acetylcholinesterase. Another advantage of the freeze-drying method is that freeze-dried vesicles can be stored and are easily reconstituted. Samples stored under an atmosphere of nitrogen at −20 °C for as long as 7 months still retained 75% of their original enzyme activity. The practical usefulness of most liposome preparations for ocular applications appears to be limited by their inherent short shelf-life [9]. Therefore, the relatively long period of stability of enzyme-containing freeze-dried vesicles upon storage becomes another added advantage.

An extremely sensitive biological test involving the induction of miosis by DFP was used to determine whether liposomes may serve as an effective carrier system for the delivery of a prophylactic agent to the eye. The prophylactic enzyme, acetylcholinesterase, binds irreversibly to DFP, which interacts chemically with a serine hydroxyl group in the enzyme active site. The rationale in using liposomes as the prophylactic carrier is at least 2-fold. Firstly, the lipid vesicles may be less susceptible to wash-out by tear flow and, secondly, if slow release of entrapped materials is required, liposomes may achieve a longer prophylactic period. Our results indicate that the application of liposome-entrapped acetylcholinesterase to the rabbit eye significantly counteracts the miotic effect induced by DFP. Positively charged liposomes appear to be more effective than neutral or negatively charged liposomes in promoting the prophylactic efficacy of the entrapped enzyme. The reason for this apparent difference is not clear. Because the corneal epithelium is thinly coated with negatively charged mucin, the positive surface charge of the liposome may provide for a more stable adsorption to the corneal surface. The enhanced adhesion of positive liposomes to the corneal surface has also been demonstrated by Schaeffer and Krohn [18] in their study of liposome-facilitated ocular drug delivery.

In an attempt to gain a better understanding of the prophylactic efficacy of liposome-entrapped enzyme, experiments were conducted to ascertain the possibility that the enzyme-liposome complex

may act like a sponge by soaking up and binding DFP molecules as well as by also releasing the entrapped enzyme to neutralize the miotic action of DFP. Our observation that tritiated DFP can be readily taken up by and released from liposomes indicated that the liposomal membranes are permeable to DFP. However, if DFP can enter the vesicles and if acetylcholinesterase is encapsulated, it is conceivable that DFP could bind to the enzyme which would effectively anchor the toxin within the liposomes. It has been shown that the retention of entrapped solutes by liposomes is best when the compounds are either very hydrophilic or very hydrophobic; substances with intermediate partition coefficients are generally diffusible unless some interaction with the bilayer occurs [19]. The diffusion of DFP across the bilayers could be expected because of its solubility in both oil and water.

Liposome-encapsulated substances usually remain within the vesicles until the liposomal membrane becomes permeable. In this study, we demonstrated that the action of tear fluid on liposomes can result in a limited release of the entrapped enzyme. The limited enzyme release observed most probably indicates exhaustion either of the enzyme available for release or of the responsible tear factor. Some of the liposome-entrapped acetylcholinesterase would be exposed on the external vesicle surface, and therefore might be more susceptible to tear-mediated removal. However, since the freeze-dried vesicles are multilamellar, the 16–22% release of enzyme (relative to controls) is probably too high to represent removal of exposed enzyme only. It is more likely that the observed release kinetics are the result of exhaustion of the tear factor(s) involved. If the latter mechanism is indeed the case, enzyme release or leakage in vivo would probably be a continuous process because of normal tear flow. This would result in different release kinetics in situ. The observation that protection is provided for at least 2 h indicates, however, that the liposomes are not rapidly destroyed because the released enzyme would then be washed away quickly. Therefore, it is most likely that the 'sponge' hypothesis is the primary mechanism of protection. The enzyme release observed when liposomes were incubated in buffer could be the result of exchange between sucrose molecules and surface-adsorbed enzyme or the results of osmotic factors when the sample was made 20% with sucrose for density-gradient centrifugation.

The data of Fig. 4 suggest that rabbit tears contain at least one substance which can increase the permeability of liposomal membranes. The rate and extent of tear fluid-induced leakage of liposome-entrapped carboxyfluorescein appear to be related to the molar ratio of cholesterol incorporated in the bilayers [20]. High cholesterol contents were found to decrease the susceptibility of liposomes to tear action. In plasma, high-density lipoprotein (HDL) is known to disrupt liposome integrity and, again, the incorporation of cholesterol can enhance the resistance to HDL-induced leakage [21–23]. Although the protein content of tears is rather low compared with that of plasma [24], a substance with HDL-like action may well be present in the tears.

This report provides the first demonstration that liposomes can serve as an effective carrier for the topical delivery of a prophylactic enzyme in counteracting chemical-induced miosis. The effectiveness of the enzyme-containing liposome appears to stem from its ability in mopping up the miotic agent and in releasing the entrapped enzyme to neutralize the agent's toxic action. Unlike other studies in which liposomes were used to facilitate the corneal penetration of entrapped drugs [18,25], the rationale of our current approach is to utilize liposomes to prolong the ocular contact time of a prophylactic agent. Therefore, the ocular penetration of the entrapped compound is not a prerequisite for successful prophylaxis. The released enzyme simply acts as a neutralizing barrier which counteracts the insult of the invading toxic agent.

Our current study reinforces the concept of using liposomes as a biocompatible carrier system for topical ocular delivery [9]. An exciting potential is the use of this approach for preventing and alleviating the irritating ocular symptoms of certain allergy sufferers to air-borne allergens. Appropriate extension of the concept embodied in this study may have far reaching implications for ocular therapy and prophylaxis.

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